32P-Postlabeling of a DNA adduct derived from 4,4 -methylenedianiline, in the olfactory epithelium of rats exposed by inhalation to 4,4 -methylenediphenyl diisocyanate

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Tissues obtained from female Wistar rats exposed to a 0.9 μm aerosol of 4,4′-methylenediphenyl diisocyanate (MDI) for 17 h per day, 5 days per week, for one year, at levels of 0, 0.3, 0.7 and 2.0 mg/m^3, were analyzed for DNA adducts. A ^{32}P-postlabeling method was used to detect (i), adducts formed by the reaction of the isocyanate group(s) of MDI with DNA; and a ^{32}P-postlabeling method was adapted to detect (ii), a DNA adduct formed by 4,4′-methylenedianiline (MDA), a hydrolysis/decarboxylation product of MDI. In the lung, neither isocyanate adducts nor the arylamine adduct were detectable. The same negative result was seen in the liver, the bladder, the kidney, the respiratory epithelium and in peripheral lymphocytes. In the olfactory epithelium, on the other hand, the arylamine-derived DNA adduct was detected, at the very low levels of 5, 9 and 10 adduct-nucleotides per 10^{10} nucleotides, for the three dose groups, respectively. The adduct co-chromatographed with the one formed in the liver of rats after oral gavage of MDA. The results are discussed in terms of the importance of genotoxic versus nongenotoxic aspects of carcinogenesis.

Introduction

4,4′-Methylenediphenyl diisocyanate (MDI*) and the corresponding diamine 4,4′-methylenedianiline (MDA) are widely used in the polymer industry. The main toxic effect of MDI in humans is respiratory sensitization (1,2). Quantification of specific antibodies to MDI covalently bound to human serum albumin assists in the diagnosis of occupational asthma induced by isocyanates (3,4). In a carcinogenicity study with Wistar rats exposed to respirable technical MDI aerosol (50% monomeric form; 93.5% of the particle sizes were below 4.2 μm in diameter), degeneration and basal cell hyperplasia of the nasal olfactory epithelium, often accompanied by hyperplasia of Bowman's glands, had been found in the 1.0 and 6.0 mg/m^3 groups. In addition, eight pulmonary adenomas (six in 60 males and two in 60 females) and one pulmonary adenocarcinoma (in a male) had been observed in the 6.0 mg/m^3 exposure group (5).

MDA induced neoplastic nodules in the liver of Fischer 344 rats and carcinomas in the liver of B6C3F1 mice, as well as thyroid carcinomas in both species in a carcinogenicity study with MDA added to the drinking water (6).

Both MDI and MDA can form macromolecular adducts. Covalent binding of MDI can occur in two ways: isocyanate adducts result from electrophilic reaction of the -N=C=O group with the nucleophilic atoms of protein and DNA whereas arylamine adducts could be formed following hydrolysis and decarboxylation of MDI to the arylamine MDA (Figure 1). Arylamines require enzymatic activation to form reactive metabolites. MDI and MDA are positive in the *Salmonella* microsome mutagenicity assay upon addition of rat liver 9000 *g* supernatant (S9) (7–9). The positive result obtained with MDI was seen only in the presence of a metabolic activation system, suggesting that the mutagenic response was due to arylamine adducts and not to isocyanate adducts.

In rat skin, isocyanate–DNA adducts were detected only at the site of topical application (10). No isocyanate adducts could be observed in liver, kidney, lung or bladder. Whether arylamine adducts had been formed was not determined, because the respective method of detection by ^{32}P-postlabeling was not available. In this report, the development of a ^{32}P-postlabeling method for the detection of MDA-derived DNA adducts is described. DNA from various tissues of female Wistar rats exposed to pure MDI aerosols (mainly monomeric form) at dose levels of 0, 0.3, 0.7 and 2.0 mg/m^3 were analyzed for isocyanate and arylamine adducts, in order to answer the question of whether DNA adduct formation represents a major mechanism of toxic action of inhaled MDI.

Materials and methods

Chemicals and enzymes

Monomeric 4,4′-methylenediphenyl diisocyanate (MDI) (Desmodur 44M) was obtained from Bayer AG (Leverkusen, Germany) and 4,4′-methylenedianiline (MDA) from Fluka AG, Buchs, Switzerland. [γ-^{32}P]ATP (7000 Ci/mmol) was supplied by ICN Biomedicals Inc (Irvine, CA). Ribonuclease A (RNase A, cat. no. R-5125), ribonuclease T1 (RNase T1, cat. no. R-1003), phosphodiesterase II (PDE, cat. no. P-6897), micrococcal nuclease (MN, cat. no. N-3755), nuclease P_1 (cat. no. N-8630) and apyrase (cat. no. A-6132) were purchased from Sigma (St Louis, MO); proteinase K (cat. no. 82945) from Fluka (Buchs, SG, Switzerland) and T4 polynucleotide kinase (PNK, cloned) from USB (Cleveland, OH). Polyethyleneimine was supplied by Fluka (Buchs, SG, Switzerland) and cellulose MN301 by Macherey and Nagel (Düren, Germany). All other chemicals were purchased either from Fluka (Buchs, SG, Switzerland) or Sigma (St Louis, MO).

Animals, treatments and tissue collection

MDI exposure. Groups of 20 female Wistar rats (Crl:[WI]BR, Charles River WIGA GmbH, Sulzfeld, Germany) were exposed in 6 m^3-whole body inhalation chambers at Fraunhofer ITA for 18 h per day nominally (on average 17 h), 5 days per week for one year to monomeric MDI aerosols. Recovery groups of 20 animals were kept in MDI-free air for 1 week after the last exposure. At the beginning of the study, the rats were 10-weeks-old with an average weight of 254 g, by the end of the study, the mean weight was 510 g. For the DNA and protein adduct formation studies (11), five rats per dose group were chosen randomly.

The mass median aerodynamic diameters of the aerosol particles had a mean value of 0.95 ± 0.12, 0.88 ± 0.13 and 0.93 ± 0.09 μm, for the three dose groups, respectively. The nominal concentrations in the inhalation chambers were 0, 0.2, 0.7 and 2.1 mg/m^3. The actual concentrations were

*Abbreviations: MDI, 4,4′-methylene diisocyanate; MDA, 4,4′-methylenedianiline; MN, micrococcal nuclease; PDE, phosphodiesterase; pd units, pixel density units; PNK, T4 polynucleotide kinase; RAL, relative adduct labeling; RNase, ribonuclease; SD, standard deviation; TLC, thin-layer chromatography.

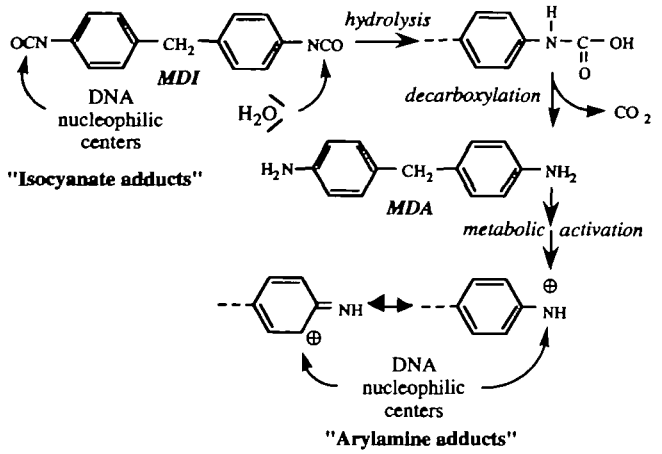

Fig. 1. The two possible pathways for the formation of DNA adducts by MDI: (left) the enzyme-independent reaction of nucleophiles with the electrophilic isocyanate group of MDI forming isocyanate adducts; (right) the enzyme-dependent reaction after hydrolysis of MDI to the carbamic acid and decarboxylation to MDA with subsequent metabolic activation to electrophilic nitrenium/carbenium ions forming arylamine adducts.

0 mg/m^3 (control), 0.26 ± 0.06, 0.69 ± 0.17 and 1.99 ± 0.40 mg/m^3. The total mass concentration in each inhalation chamber was monitored using light scattering aerosol sensors, which were calibrated by means of gravimetric analysis of filter samples. The concentration of MDI in the aerosol was determined from samples collected on PTFE filters and extracted with acetonitrile. Gaseous MDI, which according to the vapor pressure occurs at a concentration of approximately 80 µg/m^3, was collected by drawing the air sample through a glass tube filled with glass wool impregnated with the derivatizing agent, 4-nitro-*N*-propylbenzylamine (NPBA). The determination of NPBA-derivatized MDI was carried out by HPLC on a reversed phase column with acetonitrile and water as solvents. MDA was not detectable in the test atmosphere. Based on HPLC analysis, the fraction of monomeric MDI was 35.9 ± 10.6%, 81.1 ± 10.7% and 87.9 ± 4.1% for the low, middle and high aerosol concentration, respectively, including the vapor form. Details of the inhalation exposure will be published elsewhere.

The animals were killed by Nembutal i.p. and the blood was taken retro-orbitally into EDTA tubes. Peripheral lymphocytes from five animals of each dose group were isolated with Ficoll-Paque® (Pharmacia) and stored at −80°C until ^{32}P-postlabeling analysis was performed. Lung, liver, kidney, bladder and the head were immediately frozen in liquid nitrogen. The respiratory and olfactory epithelium was isolated as described (12), except that the epithelium of the endo- and exoturbinates of the ethmoid turbinate were pooled for DNA isolation. In the recovery group, more tissue was collected from the turbinates of level IV of the nasal cavity (13). Between 120–300 µg DNA were isolated from recovery group animals as compared to 70–190 µg DNA from the animals killed immediately after the end of the exposure.

MDA treatment. Three groups of three male Fischer 344 rats (~250 g) from Charles River Savo, Kisslegg, Germany were treated by gavage on three subsequent days with about 1 ml/kg body weight (b.wt) of an application solution consisting of MDA dissolved in 2 ml dimethylsulfoxide and 18 ml corn oil. The dose was 4, 20 and 50 mg MDA/kg b.wt per day. Two control rats received the vehicle only. Twenty-four hours after the third application the rats were killed with Halothane and open heart puncture. The blood was collected into heparinized tubes, peripheral lymphocytes were isolated with Ficoll-Paque® (Pharmacia) and stored at −80°C. Liver and bladder were immediately frozen in liquid nitrogen and stored at −80°C.

^{32}P-Postlabeling assay

DNA was isolated according to Beach and Gupta (14) with the following modifications. The final concentration of RNase A and RNase T1 was 166 µg/ml and 100 U/ml, respectively. Sodium dodecyl sulfate (1%) was added prior to proteinase K (200 µg/ml) treatment. Thirty µg of liver DNA from the rats treated orally with MDA were hydrolysed to the 3'-phosphates of deoxyribonucleoside with 7.5 µg MN and 7.5 µg PDE for 4.5 h at 37°C in a total reaction volume of 100 µl, whereas 15 µg DNA samples from MDI-exposed rats were digested with 5 µg MN/5 µg PDE for 3 h at 37°C. Two µl of the DNA digest were used for normal nucleotide analysis according to Gupta and Randerath (15). Adducts were enriched by the butanol procedure and the labeling reaction was performed as described (16), except that five backwashes with water were performed to ascertain that [γ-^{32}P]ATP will be in excess to the residual normal nucleotides and adducts in the labeling reaction. Adduct sensitivity for nuclease P1 was tested by the method described (14). Aliquots of 160 µCi [γ-^{32}P]ATP were used per sample in a reaction volume of 15 µl for 45 min at room temperature. Subsequent to the kinase reaction, an incubation with 1 µg apyrase was performed for 30 min. Seven µl from a total of 16 µl were applied to self-made polyethyleneimine-cellulose sheets (15). Olfactory epithelium DNA was analyzed twice. For the second run, 14 µl of the labeled solutions was used. D1 of the chromatography was run opposite to D3 with 2 M sodium phosphate, pH 6, onto 5 cm of a Whatman wick no 17. Adducts were resolved by two-directional thin-layer chromatography (TLC). Isocyanate adducts were separated in D3 and D4 by urea solvents as described (10). The arylamine adduct was subjected to development in D3 with 3.1 M lithium formate, pH 3.4, containing 5.5 M urea (LFU), followed by D4 with isopropanol: 4 M ammonia with a ratio of 0.7:1, onto 3 cm of a Whatman wick no. 1. Finally, the chromatograms were cleaned (D5) with 0.28 M ammonia, onto 3 cm of a Whatman wick no. 1. With the second analysis of olfactory epithelium DNA, a radioactivity background front was seen in the adduct region. For further removal of background radioactivity, therefore, an additional run opposite D4 was done with 0.47 M sodium phosphate, pH 8.2, 0.3 M Tris containing 4.9 M urea (SPU).

Detection and quantification of DNA adducts

Spots were either detected by autoradiography (liver of MDA-exposed rats and olfactory epithelium of rats from the recovery group exposed to MDI aerosols), and radioactivity in clearly visible spots was determined by Cerenkov counting of a cutting of the TLC plates. ^{32}P-Postlabeling analysis and quantification of DNA samples from rats exposed to MDI aerosols and the co-chromatography experiment were performed with a Phospho-Imager (BIORAD). For the calibration of the Phospho-Imager, stripes with different activities of ^{90}strontium (Sr) were prepared and exposed for 4 h. The pixel density units (pd units) of the ^{90}Sr spots were transformed to [^{32}P]-d.p.m. values using the pd units of [γ-^{32}P]ATP spots after 4 h exposure time, and whose radioactivity was determined by Cerenkov and liquid scintillation counting with a Beckman LS 6000 counter in automatic quench control mode. The ^{90}Sr-stripes were co-exposed for 4 h with all TLC-plates screened in one set and were re-used throughout the experiments. The background radioactivity was determined in cuttings of the same size and at the same place from plates used for analysis of control DNA. Cerenkov counting of the backgrounds for liver DNA were 211 ± 6 c.p.m. (1 SD). At a significance level of three SD, therefore, a minimum net DNA radioactivity of $3\times6\times\sqrt{2}$ = 25 c.p.m. would be required to be considered significant. Background counts for olfactory epithelium DNA in the recovery group were 77 ± 6 c.p.m. (1 SD). Determination of background counts with the Phospho-Imager yielded 69 ± 21 d.p.m. (1 SD). Net radioactivity was used to calculate the relative adduct labeling (RAL) = net count in adduct spot divided by the net count in the sum of the normal nucleotides, divided by the dilution factor (16). The significance level of three SD was taken for the calculation of the limit of detection. As positive controls, the arylamine adduct and the isocyanate adducts from liver DNA of MDA-treated rats and DNA incubated with MDI *in vitro* (10) were used, respectively.

The recovery of the arylamine adduct with the ^{32}P-postlabeling analysis was determined by comparison with DNA containing radiolabeled MDA adducts. A liver sample from a male Sprague–Dawley rat which had been treated intraperitoneally (i.p.) with 17.9 mg/kg [^{3}H]MDA and killed after 65 h was obtained from Dr P.Sagelsdorff, Ciba Geigy AG, Basle, Switzerland. DNA was isolated according to published methods (17) and the specific activity of 329 d.p.m./mg DNA corresponded to 70 adducts per 10^9 nucleotides. Removal of all non-covalently bound radioactivity was presumed on the basis of comparable specific activity in DNA isolated independently from the same liver and repurified to constant specific activity. Nucleotide analysis of liver DNA from [^{14}C]MDA-treated animals by HPLC had shown that all radioactivity covalently bound to DNA was recovered in an adduct fraction (to be published elsewhere). It can be assumed that the same was true for the [^{3}H]MDA-treated animals, since after normalization to the dose, the adduct levels were comparable.

Adduct standards

Chemically synthesised deoxyguanosine 3'-phosphates adducted at carbon-8 of guanine with the *N'*-acetylated, non-acetylated MDA and 4-methylaniline, i.e. *N*-(2'-deoxyguanosine-8-yl)bis(4-aminophenyl)methane, *N'*-acetyl-*N*-(2'-deoxyguanosine-8-yl)bis(4-aminophenyl)methane and *N*-(2'-deoxyguanosine-8-yl)4-methylaniline, a gift provided by Dr D.Schütze (18), were used as putative standards.

Co-chromatography

For co-chromatography of adducts, 30 µg DNA from the liver of a rat treated with 20 mg MDA/kg b.wt/day (3 ×), 30 µg DNA from the olfactory epithelium and liver of a rat exposed to 2.0 mg/m^3 MDI aerosol and 30 µg DNA from

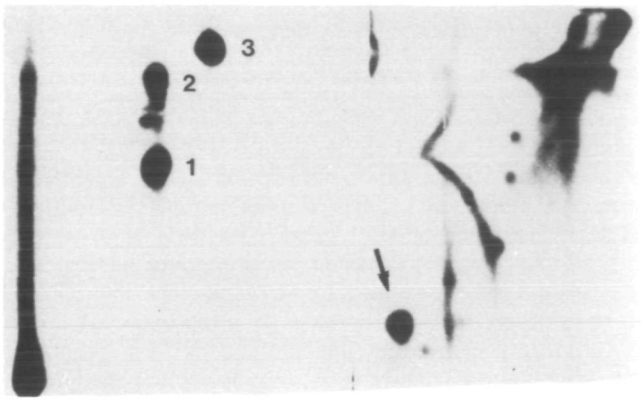

Fig. 2. ^{32}P-Postlabeling maps with a mixture of *in vitro* synthesized deoxyguanosine 3′-phosphates adducted at carbon-8 of guanine with the N′-Ac-MDA (**1**), MDA (**2**) and 4-methylaniline (**3**) (left) and the *in vivo* MDA–DNA adduct (→; right) detected in the liver of rats treated orally with MDA. The LFU/SPU solvent system was used.

the olfactory epithelium of a control rat were digested, enriched and labeled with 224 µCi [γ-^{32}P]ATP per sample. After apyrase incubation, 50 mM Tris–HCl, pH 9.5, 10 mM EDTA were added to reach a final volume of 30 µl. Thirteen µl of the ^{32}P-labeled solutions of liver DNA originating from the MDA treated rat were applied to the chromatography sheet and 13 µl of ^{32}P-labeled solution of olfactory epithelium DNA from a rat exposed to MDI were added onto the same origin.

Results

Isocyanate–DNA adducts

DNA from lung homogenates of rats exposed to MDI aerosols were analyzed by the ^{32}P-postlabeling method known to detect isocyanate–DNA adducts (10). No adduct spot was seen with a limit of detection of 0.7–3.0 adduct-nucleotides per 10^9 nucleotides, depending on the place of the adduct spots on the TLC plate. The same negative result was observed in all other tissues from these animals, i.e. in the liver, the bladder, the kidney, the respiratory epithelium and in peripheral lymphocytes.

Arylamine–DNA adduct(s)

^{32}P-Analysis for the detection of MDA-derived DNA adducts was established using liver DNA from animals treated by gavage with MDA. One adduct spot was detectable which was not identical with spots resulting from the labeling of the three *N*-(deoxyguanosine-8-yl) arylamine adducts (Figure 2). In the LFU/SPU solvent system, the region of the *in vivo* arylamine adduct lies within a diagonal radioactive zone, causing great variation in the background levels. In order to reduce background radioactivity, the D4 urea solvent was replaced by ammonia/isopropanol, resulting in the low limit of detection of 3 adduct-nucleotides per 10^{10} nucleotides with liver DNA. The respective RAL values were in linear proportion to the dose applied (Figure 3). Nuclease P1 treatment resulted in complete loss of this adduct, i.e. the MDA–DNA adduct is nuclease P1-sensitive like most other arylamine adducts (14). ^{32}P-Postlabeling analysis of DNA isolated from the bladder and from lymphocytes of these animals did not show any treatment-related DNA-adduct spots.

In order to investigate what fraction of the total number of MDA–DNA adducts could be detected with this single spot, a ^{32}P-postlabeling analysis was also performed on a DNA sample with an adduct level of 70 adducts per 10^9 DNA nucleotides that was isolated from the liver of an animal that had been treated with tritiated MDA. The ^{32}P-postlabeling analysis of this DNA resulted in a RAL value of 1.1 ± 0.5 adduct-nucleotides per 10^9 DNA nucleotides. Therefore, the recovery of the single detectable adduct was only 1–2% of the DNA adducts present.

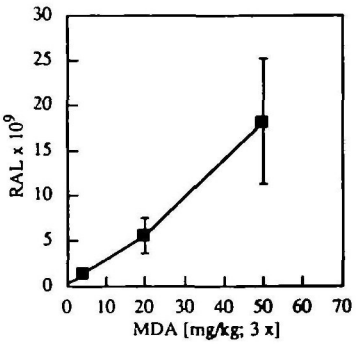

Fig. 3. Adduct concentrations (RAL values) determined for one DNA-adduct spot in liver DNA of male Fischer 344 rats treated by gavage with 4, 20 and 50 mg MDA/kg b.wt on three subsequent days and killed 24 h after the last dose. Means ± 1 SD (n = 3). At 4 mg/kg, the SD was smaller than half the symbol size.

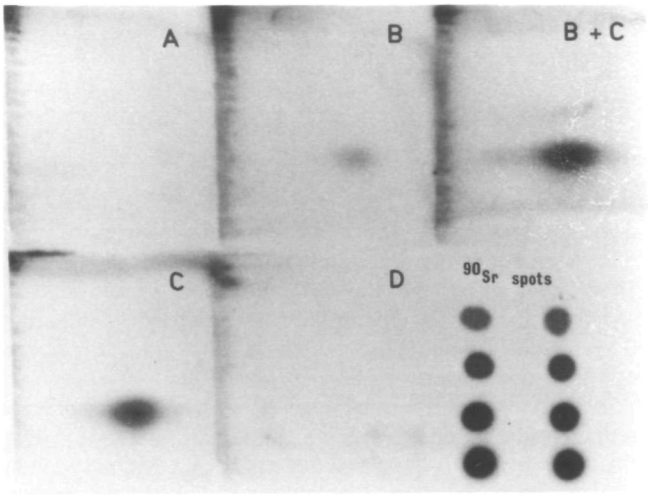

Fig. 4. (**A**) ^{32}P-postlabeling maps of DNA isolated from the olfactory epithelium of a control rat of the MDI aerosol study; (**B**) from the olfactory epithelium of a rat exposed to 2.1 mg/m^3 MDI aerosol for 1 year; (**C**) from the liver of a MDA-treated rat; (**D**) the liver of the same animals as in (B). Co-chromatography plate of (B + C). Calibration of the Phospho-Imager by ^{90}Sr spots which had been calibrated with ^{32}P.

In the lung of rats exposed to MDI aerosol, i.e. the target organ for MDI induced tumors, no MDA–DNA adduct was detectable. However, this adduct was seen at very low levels in DNA of the olfactory epithelium. Co-chromatography of DNA from the olfactory epithelium of MDI-exposed rats with liver DNA from MDA-treated animals confirmed the co-migration of the spots on the TLC-plate. The respective postlabeling maps are shown in Figure 4. The adduct concentration increased with dose in a supralinear manner (Figure 5). At the lowest dose of 0.3 mg/m^3, which is only six times the actual threshold limit value of 5 ppb, adduct spots were detectable at concentrations of 5 ± 1 adduct-nucleotides per 10^{10} nucleotides. In the 0.7 mg/m^3 dose group RAL values were 9 ± 4 adduct-nucleotides per 10^{10} nucleotides, and in the high dose group 10 ± 3 adduct-nucleotides per 10^{10} nucleotides. Since all DNA samples have been postlabeled in one set, the interanimal variation should have been independent on the labeling efficiency. Unexpectedly, the recovery group

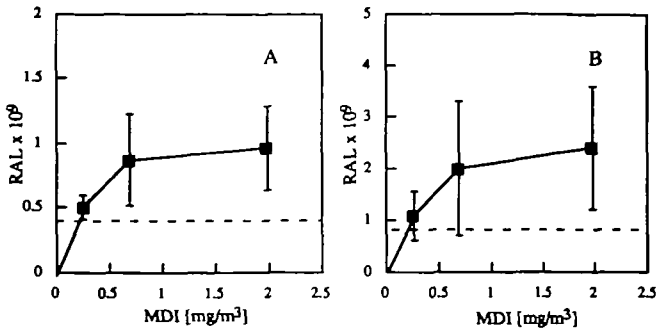

Fig. 5. Adduct concentrations (RAL values) determined in DNA isolated from the olfactory epithelium of female Wistar rats exposed to MDI aerosol for 1 year, without (**A**) and with (**B**) 1 week of exposure-free recovery. Means ± SD (n = 5); dashed line: limit of detection.

showed higher adduct concentrations. This could be related to either the more extensive collection of tissue from the turbinates, which could contain highly adducted DNA and/or to a decrease in cytotoxicity after stopping the exposure. Regenerative cell division after cytotoxicity is associated with a higher rate of DNA replication resulting in a dilution of the DNA adduct concentrations. The limit of detection in olfactory epithelium DNA was 4 adduct-nucleotides per 10^{10} nucleotides, and in olfactory epithelium DNA from the recovery group 8 adduct-nucleotides per 10^{10} nucleotides.

In all other organs investigated from MDI-exposed rats (i.e. in the liver, the bladder, the kidney, the respiratory epithelium and peripheral lymphocytes) no MDA–DNA adduct spot was above the limit of detection.

All DNA samples from a given tissue were analyzed simultaneously together with an aliquot of a standard liver DNA sample from a rat treated with 50 mg MDA/kg. RAL values of these positive controls were 2.1 ± 1.4 adduct-nucleotides per 10^8 nucleotides (n = 13), i.e. the coefficient of variation for the independent postlabeling analyses was 66%.

Discussion

We had previously established a ^{32}P-postlabeling method to detect (i) DNA adducts derived from the action of the isocyanate groups of MDI with DNA (10). In this study, the development of a ^{32}P-postlabeling assay is demonstrated for the analysis of a DNA adduct formed with MDA, the arylamine resulting from hydrolysis and decarboxylation of MDI with subsequent metabolic activation to reactive intermediates. One dose-dependent adduct spot was observed in liver DNA from rats treated by gavage with MDA. Interestingly, this MDA–DNA adduct was not identical with the *N*-(deoxyguanosine-8-yl) arylamine derivates, which are the predominant DNA adducts formed by the majority of arylamines (19). The recovery of this single detectable adduct was 1–2% of the adducts formed in liver DNA on the basis of [^{3}H]adduct radioactivity after administration of [^{3}H]MDA. This recovery rate should not be used for the calculation of the total adduct concentration in chronically exposed animals. In view of adduct-specific rates of repair, adduct profiles after acute dosing are expected to differ from those seen under steady-state conditions reached with chronic exposure.

In the rats treated chronically by inhalation with MDI aerosol, DNA-adduct formation was detectable exclusively in the nasal olfactory epithelium. In this tissue, degeneration and basal cell hyperplasia often accompanied by hyperplasia of Bowman's glands, had been found in animals exposed by inhalation to technical MDI aerosol (5). Rather unexpected, the adduct formed was not an isocyanate adduct but an arylamine DNA adduct. It is conceivable that the aerosol particles and MDI in vapor phase could have been trapped within the architecture of the ethmoid turbinates. Hydrolysis and decarboxylation of MDI to MDA is likely to occur when the MDI-loaded air is moistened in the upper respiratory tract. Bioactivation of arylamines to DNA-reactive intermediates had been observed in organs other than the liver (20,21), but DNA-adduct formation by procarcinogens in the olfactory epithelium had been shown so far only for polycyclic aromatic hydrocarbons such as benzo[*a*]pyrene (22) and for nitrosamines such as the tobacco specific 4-(*N*-methyl-*N*-nitrosamino)-1-(3-pyridyl)-1-butanone (23–25).

The dose–response curves for the MDA–DNA adduct in the olfactory epithelium was supralinear. Interestingly, in the same animals, hemoglobin adduct levels showed the same shape of the dose–response curve (11). The ratio of protein adduct formation in erythrocytes and DNA adduct formation in a target tissue for toxicity, therefore, appeared to be constant over the dose range tested.

The question arises why hemoglobin adducts were detectable but not systemic DNA adduct formation. Three points might explain the discrepancy: (i) protein contain stronger nucleophilic centers than DNA, resulting in a faster rate of adduct formation, (ii) hemoglobin adducts are not repaired, and (iii) the lifetime of erythrocytes is longer than the lifetime of most cell types, thus allowing an accumulation of adducts over longer periods of time.

In the lung, the target organ for tumor formation in the rat, after inhalation exposure to technical MDI (5), no DNA-adduct was detectable. This was unexpected in view of the data with rat skin, where topically administered MDI (3 mg/cm^2) had been shown to reach DNA of epidermal cells and form isocyanate–DNA adducts (10). The following considerations could account for the discrepancy. First, due to the high electrophilic reactivity of the isocyanates, only cells lining the epithelium of the lung would be adducted by MDI. DNA was isolated from lung homogenates so that potentially high adduct concentrations in the cells close to the airways were diluted by more remote cells. It would therefore be necessary to study cell subpopulations in order to determine adduct concentrations in cells at risk. Second, the surface load could have been too low in the lung to form detectable adduct concentrations. It was about 80-fold lower in lung when compared with the olfactory epithelium, assuming 15% deposition of 1 µm particles in the lung (26) with a surface area of 3900 cm^2 (27) and a 2% deposition in the skull and an olfactory epithelium surface area of 6.6 cm^2 (28). Third, the pathway for diffusion of the MDI aerosol is longer in the bronchiolar/alveolar region of the lung as compared to the turbinates of the olfactory epithelium, leading to more pronounced hydrolysis/decarboxylation of MDI to MDA in the lung. Since MDI is able to react with MDA to form mixed ureas, the addition reaction occurring at the outer surface of the aerosol can result in the formation of chemically inert particles.

Lung tumor formation in the rat has been observed not only upon exposure to DNA-reactive carcinogens, but also to chemically inert particles. Comparison of carcinogenic potency of chronically inhaled diesel exhaust and carbon black had indicated that the organic fraction did not play the major role

in the carcinogenicity of diesel exhaust in the lung of rats (29). Although MDI-derived particles are expected to act differently in the lung due to the different chemical composition, it is conceivable that factors such as chronic inflammatory response, cell injury and cell proliferation influenced the formation of tumors by MDI.

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References

1. Karol,M.H. (1986) Respiratory effects of inhaled isocyanates. *CRC Crit. Rev. Toxicol.*, **16**, 349–379.
2. Schnorr,T. and Egilman,D. (1988) Toluene diisocyanate and other isocyanates. In Zenz,C (ed.), *Occupational Medicine; Principles and Practical Applications*. Year Book Medical Publishers, Chicago, pp. 1016–1021.
3. Vandenplas,O., Malo,J.-L., Dugas,M., Cartier,A., Desjardins,A., Lévesque,J., Shaughnessy,M.A. and Grammer,L.C. (1993) Hypersensitivity pneumonitis-like reaction among workers exposed to diphenylmethane diisocyanate (MDI). *Am. Rev. Respir Dis.*, **147**, 338–346.
4. Bernstein,D.I., Korbee,L., Stauder,T., Bernstein,J.A., Scinto,J., Herd,Z.L. and Bernstein,I.L. (1993) The low prevalence of occupational asthma and antibody-dependent sensitization to diphenylmethane diisocyanate in a plant engineered for minimal exposure to diisocyanates. *J. Allergy Clin. Immunol.*, **92**, 387–396.
5. Reuzel,P.G.J., Arts,J.H.E., Lomax,L.G., Kuijpers,M.H.M., Kuper,C.F., Gembardt,C., Feron,V.J. and Löser,E. (1994) Chronic inhalation toxicity and carcinogenicity study of respirable polymeric methylene diphenyl diisocyanate (polymeric MDI) aerosol in rats. *Fund. Appl. Toxicol.*, **22**, 195–210.
6. IARC (1986) 4,4′-Methylenedianiline and its dihydrochloride. In *IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans: Some Chemicals Used in Plastics and Elastomers*. IARC Scientific Publication, Lyon, no. 39, pp. 347–365.
7. Andersen,M., Binderup,M.-L., Kiel,P., Larsen,H. and Maxild,J. (1980) Mutagenic action of isocyanates used in the production of polyurethanes. *Scand. J. Work Environ. Health*, **6**, 221–226.
8. Shimizu,H., Suzuki,Y., Takemura,N., Goto,S. and Matsushita,H. (1985) The results of microbial mutation tests for forty-three industrial chemicals. *Jpn J. Ind. Health*, **27**, 400–419.
9. Zeiger,E., Anderson,B., Haworth,S., Lawlor,T., Mortelmans,K. and Speck,W. (1987) *Salmonella* mutagenicity tests: III. Results from the testing of 255 chemicals. *Environ. Mutagenesis*, **9**, 1–110.
10. Vock,E.H., Cantoreggi,S., Gupta,R.C. and Lutz,W.K. (1995) ^{32}P-Postlabeling analysis of DNA adducts formed *in vitro* and in rat skin by methylenediphenyl-4,4′-diisocyanate. *Toxicol. Lett.*, **76**, 17–26.
11. Sepai,O., Schütze,D., Heinrich,U., Hoymann,H.G., Henschler,D. and Sabbioni,G. (1995) Hemoglobin adducts and urine metabolites of 4,4′-methylenedianiline after 4,4′-methylenediphenyl diisocyanate exposure of rats. *Chem. -Biol. Interactions*, **97**, 185–198.
12. Kuykendall,J.R., Taylor,M.L. and Bogdanffy,M.S. (1993) Cytotoxicity and DNA-protein crosslink formation in rat nasal tissues exposed to vinyl acetate are carboxylesterase-mediated. *Toxicol. Appl. Pharmacol.*, **123**, 283–292.
13. Uraih,L.C. and Maronpot,R.R. (1990) Normal histology of the nasal cavity and application of special techniques. *Environ. Health Perspect.*, **85**, 187–208.
14. Beach,A.C. and Gupta,R.C. (1992) Human biomonitoring and the ^{32}P-postlabeling assay. *Carcinogenesis*, **13**, 1053–1074.
15. Gupta,R.C. and Randerath,K. (1988) Analysis of DNA adducts by ^{32}P labeling and thin layer chromatography. In Friedberg,E.C. and Hanawalt,P.C. (eds), *DNA Repair*. Marcel Dekker, Inc., New York, 3, pp. 399–418.
16. Gupta,R.C. (1985) Enhanced sensitivity of ^{32}P-postlabeling analysis of aromatic carcinogen: DNA adducts. *Cancer Res.*, **45**, 5656–5662.
17. Sagelsdorff,P., Lutz,W.K. and Schlatter,C. (1983) The relevance of covalent binding to mouse liver DNA to the carcinogenic action of hexachlorocyclohexane isomers. *Carcinogenesis*, **4**, 1267–1273.
18. Schütze,D., Sabbioni,G. and Sagelsdorff,P. (1993) Synthesis and [^{32}P]-postlabelling of DNA-adducts of 4,4′-methylenedianiline (MDA). *Naunyn-Schmiedeberg's Arch Pharmacol.*, **347** (Suppl.), R20.
19. Kadlubar,F.F. (1994) DNA adducts of carcinogenic aromatic amines. In Hemminki,K., Dipple,A., Shuker,D.E.G., Kadlubar,F.F.,Segerbäck,D. and Bartsch,H. (eds), *DNA Adducts: Identification and Biological Significance*. IARC Scientific Publications, Lyon, no. 125, pp. 199–215.
20. Devereux,T.R., Domin,B.A. and Philpot,R.M. (1993) Xenobiotic metabolism by isolated pulmonary bronchiolar and alveolar cells. In Gram,T.E. (ed.), *Metabolic Activation and Toxicity of Chemical Agents in Lung Tissue and Cells*. Pergamon Press, New York, pp. 25–40.
21. La,D.K. and Froines,J.R. (1994) Formation and removal of DNA adducts in Fischer-344 rats exposed to 2,4-diaminotoluene. *Arch. Toxicol.*, **69**, 8–13.
22. Bond,J.A., Wolff,R.K., Harkema,J.R., Mauderly,J.L., Henderson,R.F., Griffith,W.C. and McClellan,R.O. (1988) Distribution of DNA adducts in the respiratory tract of rats exposed to diesel exhaust. *Toxicol. Appl. Pharmacol.*, **96**, 336–346.
23. Belinsky,S.A., Walker,V.E., Maronpot,R.R., Swenberg,J.A. and Anderson,M.W. (1987) Molecular dosimetry of DNA adduct formation and cell toxicity in rat nasal mucosa following exposure to the tobacco specific nitrosamine 4-(*N*-methyl-*N*-nistrosamino)-1-(3-pyridyl)-1-butanone and their relationship to induction of neoplasia. *Cancer Res.*, **47**, 6058–6065.
24. Hecht,S.S., Carmella,S.G., Foiles,P.G., Murphy,S.E. and Peterson,L.A. (1993) Tobacco-specific nitrosamine adducts: Studies in laboratory animals and humans. *Environ. Health Perspect.*, **99**, 57–63.
25. Trushin,N., Rivenson,A. and Hecht,S.S. (1994) Evidence supporting the role of DNA pyridyloxobutylation in rat nasal carcinogenesis by tobacco-specific nitrosamines. *Cancer Res.*, **54**, 1205–1211.
26. Raabe,O.G., Al-Bayati,M.A., Teague,S.V. and Rasolt,A. (1988) Regional deposition of inhaled monodisperse coarse and fine aerosol particles in small laboratory animals. *Ann. Occup. Hyg.*, **32**, 53–63.
27. Gil,J. (1982) Comparative morphology and ultrastructure of the airways. In Witschi,H.P. and Nettesheim,P. (eds), *Mechanisms in Respiratory Toxicology*. CRC Press Inc., Boca Raton, 1, pp. 3–25.
28. Gross,E.A., Swenberg,J.A., Fields,S. and Popp,J.A. (1982) Comparative morphometry of the nasal cavity in rats and mice. *J. Anatomy*, **135**, 83–88.
29. Nikula,K.J., Snipes,M.B., Barr,E.B., Griffith,W.C., Henderson,R.F. and Mauderly,J.L. (1995) Comparative pulmonary toxicities and carcinogenicities of chronically inhaled diesel exhaust and carbon black in F344 rats. *Fund. Appl. Toxicol.*, **25**, 80–94.

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